TITLE OF THE INVENTION

Packaging cells for recombinant adenovirus

FIELD OF THE INVENTION

The invention relates to the field of molecular and cell biology, more in particular to packaging cells and use thereof for the generation of batches of recombinant adenovirus.

BACKGROUND OF THE INVENTION

Replication deficient recombinant adenoviruses are useful for instance in gene therapy and for vaccination purposes. They usually lack the E1-region of an adenovirus, and are therefore propagated on complementing cells providing the E1 sequences. The packaging cells provide all information required for replication of a vector that is packaged in the cells to form recombinant viral particles. An example of a packaging cell is a 293 cell, which contains nucleotides 1-4344 of the adenovirus 5 genome (Louis et al, 1997), which include the 5' inverted terminal repeat (ITR), the packaging signal, the E1A and E1B coding sequences and the pIX coding sequences. Overlap between the adenoviral sequences in the packaging cell and the vector may lead to homologous recombination between these sequences, resulting in the generation of replication competent adenovirus (RCA) (Lochmuller et al., 1994). This problem has been solved by using a packaging system consisting of a cell line and a vector, that are matched to each other by lacking such overlapping sequences (Fallaux et al, 1998; US patent 5,994,128). One example of a particularly useful cell line in such applications is the PER.C6® cell line (US patent 5,994,128; Nichols et al, 2002).

Recently, it was reported that upon use of PER.C6® cells in conjunction with a vector that still contained 177 bp sequence homology with the E1 sequences in the genome of PER.C6®, a single cross over event could result in the generation of helper dependent replication competent virus at a low frequency (Murakami et al, 2002). The generated atypical RCA was termed helper-dependent E1 containing particle (HDEP). As expected, the emergence of this type of particle was circumvented when a vector was used that lacked the sequence overlap with the E1 sequences in the genome of PER.C6® cells (Murakami et al, 2002).

However, it now unexpectedly appears that, using the system of matched vector and PER.C6® cells, some batches of recombinant adenovirus are contaminated at a very low frequency with particles that can cause cytopathological effect (CPE) in cells lacking E1 sequences. The particle is helper-dependent and contains E1-sequences, and therefore is also an HDEP. This HDEP is generated in the absence of substantial homology between vector and packaging cell (see Murakami et al, 2004). While RCA is recognized by the regulatory authorities as a potential problem and detection of RCA in batches for clinical use is mandatory (USDHHS, FDA, CBER. Guidance for Industry, Guidance for human somatic cell therapy and gene therapy, March 1998), the safety aspects of HDEP are unclear. HDEP is replication deficient since it lacks the necessary viral genes for autonomous replication and therefore HDEP will not disseminate in a host. The theoretical possibility that the presence of a recombinant vector or wild-type adenovirus in the same cell may cause rescue and spread of HDEP, can however not be completely excluded. Hence, there is at least a latent need for means and methods for the prevention of generation of E1-containing particles during the preparation of batches of

recombinant adenovirus particles. To this purpose, the present invention provides methods for preparing new cell lines, new cell lines, and use thereof to make batches of RCA-free and HDEP-free recombinant adenovirus vectors.

DESCRIPTION OF THE FIGURES

Fig. 1: Map of pIG.E1A

Fig 2: Map of pIG.E1AB21

Fig 3: Map of pE1B

Fig 4: Map of pCC.E1A

Fig 5: Map of pCC.E1AB21

Fig 6: Map of pCC101

Fig 7: Map of pCC105

Fig 8: Map of pCC.55Kcol

Fig 9: Map of pIG.E1B

Fig 10: Map of pCC.E1Bcol

Fig 11: Map of pEC.E1B

Fig 12: Map of pSC.55K

Fig 13: Example of the design of large molecules suitable for the transformation of primary cells.
I: schematic presentation of single cosmid vector with coding regions for E1A and E1B proteins (A and B respectively) flanked by spacer DNA. RE= restriction enzyme recognition site not present in the spacer DNA or E1 sequences.
II: schematic representation of two separate nucleic acids (e.g plasmids or cosmid vector) having either E1A (A) or E1B (B) coding regions flanked by spacer DNA
III: Situation that may occur after transformation and integration into host cell genome of a primary cell with the molecule from I or with the two molecules of II. In the host genome the insert consists of an E1A region and an E1B region that are separated by spacer DNA and a second copy of that fragment / those fragments in inverted orientation. J: junction between inverted repeat. If a part of these sequences (indicated by arrows) recombine in the recombinant adenovirus the genome becomes too large to be packaged. ITR: inverted terminal repeat, Φ: packaging signal, FI: Fragment Inserted into recombinant virus, here including inverted repeat sequences. Although the presence of an inverted repeat may help in the subsequently necessary step of deleting other sequences from the virus genome, the presence of an inverted repeat sequence results in a mirror-imaged duplication of the left end of the recombined virus during replication of the virus genome and generates a genome (duplicated fragment, DF) that again is too large to be packaged due to the spacer DNA.

Fig 14: Map of pIG.E1A.E1B

Fig 15: Map of pCC200

Fig 16: Map of pCC205

Fig 17: Map of pdys44

Fig 18: Map of p44-1.ccE1A

Fig 19: Western blots of protein lysates from generated cell lines. Lanes: 1: HER01-B-71, 2: HER01-H-86, 3: HER01-H-87, 4: HER01-H-88, 5: HER01-H-89, 6: HER01-B-90, 7: HER01 pn06 cells, 8: PER.C6 cells

Fig 20: Demonstration of complementation of E1-deleted adenovirus vectors with transformed cell clones. -: negative control; MOF: mean of fluorescence; B-71, B-90, H-86, H-87, H-88, H-89: generated transformed cell clones

Fig 21: Schematic overview of the Ad5.E1 constructs used for the transfection in example 3. The restriction sites used to digest the genomic DNA and to generate the probe fragments are indicated. The E1A and the E1B DNA fragment are fictively linked with nucleotide numbering starting at the 5′ end of the E1A-containing fragment.

Fig 22: Schematic presentation of some embodiments of the invention (not to scale). Regulating sequences (such as promoters and polyA signals) are not depicted.
A. natural configuration of the E1-region of the adenovirus genome: E1A is followed by E1B, which consists of E1B-19K and E1B-55K. No spacer sequence is present between E1A and E1B. Packaging cells of the prior art have been constructed by introducing DNA in this configuration, and hence also have the E1 region in this configuration in their genome.

B. The two simplest of the possible configurations according to the present invention where a spacer (or stuffer) is present in the E1 region. 1. Stuffer introduced between E1A and E1B-19K. 2. Stuffer introduced between E1B-19K and E1B-55K. The rationale is to increase the distance between E1A, E1B-19K and E1B-55K compared to the natural situation, to decrease the possibility or prevent altogether the simultaneous uptake of these three open reading frames into a single adenovirus particle (prevent RCA and HDEP). The stuffer should be at least 4 kb to obtain a cell line according to the invention, said cell comprising adenovirus E1A and E1B-55K and E1B-19K coding sequences in its genome, characterized in that said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 4 kb in said genome. The order of the open reading frames does not matter as long as they are all present in the genome in a way that there is expression of E1A, E1B-19K and E1B-55K. Hence the order may E1A-E1B/19k-E1B/55k, E1B/19k-E1B/55k-E1A, E1B/55k-E1B/19k-E1A, E1B/55k-E1A-E1B/19k or E1B19k-E1A-E1B55k. When, the E1B/19k and the E1B/55k are adjacent they may be present as a single transcription unit or as separate transcription units, this is not critical to the invention. Both configurations (1 and 2) will be able to generate cell lines that fulfil this spacing criterion of the invention. Stuffer lengths of at least 6, 8, 10, 15 or 34.5 kb can generate cells according to the invention having all three open reading frames but lacking stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 6, 8, 10, 15 or 34.5 kb in their genome. This means that when a single fragment containing E1A + E1B-19K + E1B-55K together should come from the genome of the generated cells, this fragment should at least be 7 kb

(stuffer 4 kb), 9 kb (stuffer 6 kb), etc, until at least 37.5 kb (stuffer of 34.5 kb), in contrast to the situation in A, where these sequences can be found in a fragment of only 3 kb of the genome of packaging cells generated with such constructs (E1A + E1B-19K + E1B-55K open reading frames together comprise about 3 kb).

C. Extension of principle of B: since the fragments as shown in B could integrate adjacent to each other, a stuffer is also placed before E1A and/or after E1B in preferred embodiments of the invention (prevents close proximity of E1B of first repeat to E1A of second repeat). The genome of a resulting packaging cell is shown, wherein two copies of the E1 region provided with stuffer DNA are shown when integrated adjacent to each other in tandem repeat. These packaging cells of the invention have integrated in their genome E1A and E1B-19K and E1B-55K, but do not contain stretches of nucleic acid in their genome wherein said E1A and both E1B coding sequences are separated by less than 4 kb, etc, depending on the stuffer lengths (should in this example be at least 4 kb between E1A and E1B-19K (no. 1) or between E1B-19K and E1B-55K (no. 2), and at least 2 kb upstream of E1A and 2 kb downstream of E1B-55K (together 4 kb when integrated directly adjacent)).

DESCRIPTION OF THE INVENTION

The invention describes methods to generate a packaging cell comprising adenoviral E1A and E1B sequences, wherein E1A and at least one of the E1B coding regions are separated from each other in the genome of the cell. To this purpose, at least three embodiments are provided. In a first embodiment, E1A and E1B can be introduced in a precursor cell on different moments in time, reducing the chance that E1A and E1B are co-

integrated on the same chromosomal location. In a second embodiment, the chances of co-integration into the same locus are reduced by introducing E1A and E1B coding sequences into a precursor cell on two different molecules that lack overlapping sequences which could otherwise lead to homologous recombination between these sequences. In a third embodiment, E1A and at least one of the E1B coding regions are introduced by virtue of one nucleic acid molecule whereon these sequences are separated by a given distance. It will be clear that in the third embodiment, instead of a single nucleic acid molecule also two or more nucleic acid molecules can be introduced, which would satisfy the criterion that the E1A and at least one of the E1B coding regions are separated by a given distance, in case these two or more molecules would form one molecule, e.g. by ligation, recombination, and the like. The resulting packaging cells are characterized in that E1A and both E1B coding regions are present at a distance from each other, and hence the chances of E1A and both E1B coding sequences together being incorporated into the genome of a recombinant adenovirus that is propagated on such packaging cells are reduced.

The invention provides a method of preparing a cell capable of complementing E1 deficient adenoviral vectors without generating helper dependent E1 containing particles, comprising the steps of: a) introducing into a precursor or ancestor of the cell nucleic acid sequence(s) coding for E1A gene functions and E1B gene functions, or if the precursor cell already comprises one of E1A or E1B gene functions, the other of the E1A and E1B gene functions; and b) selecting or identifying cells having obtained E1A and E1B in a chromosomal configuration that prevents the formation of helper dependent E1 containing particles when the cells are used to complement

a recombinant adenoviral vector deficient for one or more E1 gene functions, said vector lacking nucleic acid sequences that have substantial overlap with the chromosomally located E1 sequence that otherwise could give rise to homologous recombination.

In one aspect, the invention provides a method of preparing a cell having adenovirus E1A and E1B-19K and E1B-55K coding sequences in its genome, wherein nucleic acid sequences comprising said sequences are introduced into a precursor cell, the method characterized in that: a) at least two nucleic acid molecules together comprising said sequences are introduced into the precursor cell, wherein said E1A and at least one of the E1B coding sequences are present on different nucleic acid molecules, which are introduced into the precursor cell on a different moment in time, and wherein said precursor cell is not a baby rat kidney cell; or b) a nucleic acid molecule comprising the E1A coding sequence and a nucleic acid molecule comprising the E1B coding sequences are introduced into the precursor cell, wherein said nucleic acid molecules lack substantial overlap that could otherwise lead to homologous recombination between said at least two molecules; or c) said nucleic acid sequences comprising E1A and at least one of the E1B coding sequences are separated by at least 4 kb on a single nucleic acid molecule or on two or more nucleic acid molecules when these would form a single nucleic acid molecule. Preferably, in embodiment c) said sequences are separated by at least 10 kb, more preferably at least 34.5 kb or more. Preferably the separating sequences are non-E1 sequences, so that integration of both expression cassettes of E1A and E1B together in a single virus genome is rendered very unlikely or impossible. Alternatively, when the E1A and at least one of the E1B sequences are separated by 0-

15 kb, a screen for the absence of inverted repeats of the E1-sequences in the generated cells may be used to select for cells wherein the possibility of generating HDEP upon propagation of recombinant adenovirus in the generated cells is decreased or absent. The invention also provides cells obtainable by the method according to the invention. The invention further provides a cell comprising adenovirus E1A and E1B-55K and E1B-19K coding sequences in its genome, characterized in that said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 4 kb in said genome. Preferably said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 10 kb in said genome, more preferably said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 34.5 kb in said genome.

It is another aspect of the invention to provide a method for providing or generating a cell comprising adenoviral E1 sequences, wherein said E1 sequences include E1A and E1B coding sequences, characterized in that said method includes a step of selecting cells lacking inverted repeats comprising said E1 sequences. The invention further provides a cell comprising adenovirus E1 sequences in its genome, wherein said E1 sequences include E1A and E1B coding sequences, characterized in that said E1 sequences are not present in the form of inverted repeats in said genome. In one embodiment thereof, said cell comprises at least two copies of said adenovirus sequences in its genome, which copies may be on the same chromosome. In another embodiment thereof, said cell comprises one copy of said E1 sequences in its genome, and further lacks sequences in its genome encoding (functional)

pIX of an adenovirus.

It is another aspect of the invention to provide a method for generating a batch of recombinant adenovirus having a deletion in the E1 region, comprising the steps of: a) introducing said recombinant adenovirus into a cell comprising E1 sequences of an adenovirus capable of complementing the deleted E1 sequences of said recombinant adenovirus; b) culturing said cell and harvesting said recombinant adenovirus, the method characterized in that said cell is a cell according to the invention. It will be clear to the person skilled in the art that instead of introducing said recombinant adenovirus, it is also possible to start the generation of recombinant adenovirus by using one or more nucleic acid fragments capable of forming the genome of said recombinant adenovirus, and which will after replication and packaging in said cell form said recombinant adenovirus. Hence, for this embodiment, a recombinant adenovirus may also be a genome of the recombinant adenovirus. In a preferred embodiment, said recombinant adenovirus lacks substantial sequence overlap with the E1 sequences present in said cell, which could otherwise lead to homologous recombination. In yet another embodiment, the invention provides a packaging system comprising a packaging cell comprising E1 sequences in its genome and a recombinant adenovirus vector with a deletion in the E1 region, wherein the genome of said vector lacks substantial overlap with the E1 sequences in the genome of said packaging cell, characterized in that said packaging cell is a cell according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A replication competent adenovirus (RCA) is an adenoviral particle that is capable of replication in cells without the need for helper adenovirus. An 'HDEP' (helper dependent E1-containing particle) as used herein is defined as a viral particle comprising at least E1A and E1B-55K coding sequences from an adenovirus, said particle not being able to replicate in a cell lacking functional E1A and/or E1B-55K in the absence of 'helper virus'. A helper virus may be wild type or mutant adenovirus but also any adenoviral vector providing the functions missing in HDEP, and can therefore be provided by the (E1 defective) desired product recombinant adenovirus propagated on the packaging cell. The E1A and E1B 55K sequences are required for propagation of an adenovirus. The HDEP usually further contains E1B-19K coding sequences. The E1 sequences present in HDEP should be able to complement the recombinant E1-defective adenovirus, which can serve as a helper virus for HDEP. HDEP can be generated by homologous recombination on overlap between the E1 sequences in a packaging cell and a vector (Murakami et al, 2002), but as now unexpectedly appears from several studies, it can also be generated in the absence of substantial homology/overlap between sequences in the packaging cell and vector, i.e. by a process referred to herein as non-homologous recombination (see e.g. Murakami et al, 2004 for possible structures of HDEP). 'Substantial overlap' as used herein is therefore defined as sufficient overlap for homologous recombination. In any case in embodiments where the invention refers to 'absence of substantial overlap', this condition is considered to be fulfilled if an overlap exists of no more than 50 nucleotides (nt), preferably less than 40 nt, more preferably less than 30 nt, still more preferably less than 20 nt, still more preferably less than 10 nt, most preferably no overlap at all.

Although the mechanism of the formation of HDEP is not fully understood at present, it is clear that the E1 sequences therein originate from the genome of the packaging cell. The presence of E1 sequences in recombinant adenovirus batches is unwanted, being it in the form of RCA or HDEP. The invention provides methods and means that minimize the chance of generating HDEP. Thereto, methods for preparing cells and the resulting cells are provided, wherein said cells have E1A and E1B in a chromosomal configuration that prevents the introduction of E1A and E1B coding sequences together into a recombinant adenovirus, and hence the formation of HDEP when the cells are used for the complementation of E1-deficient recombinant adenoviral vectors, in the absence of substantial sequence overlap between the vector and the chromosomally located E1 sequences of the complementing cells of the invention. To this purpose, the chromosomal conformation of the cells according to one embodiment of the invention is such that E1A and at least one of the E1B coding regions are separated in the genome of the novel cells. Since inverted repeats are believed to contribute to the formation of HDEP by stimulating the deletion of adenovirus sequences, in another embodiment the invention provides cells comprising E1A and E1B coding sequences, wherein the E1 sequences are not present in inverted repeat conformation in the genome of said packaging cells.

The cells of the invention comprise adenovirus E1A and E1B coding sequences in their genome, and preferably they comprise the coding sequences for all functional E1A and both E1B (E1B-55K and E1B-19K) proteins in their genome. E1A and at least one of the E1B proteins may be of the same or optionally be of different serotype according to the invention.

Preferably in these embodiments, at least one of E1A and E1B are regulated by a promoter different from an E1A and E1B promoter, respectively.

Cells of the invention.

A recombinant adenovirus may be able to take up >20 kb of DNA from the cell line in which it is propagated, and the maximum genome length of an adenovirus allowing efficient packaging is approximately 38 kb (for an adenovirus type 5 based vector; this figure may depend somewhat on the serotype). This feature is used to prepare novel complementing cells according to the invention, said cells comprising E1A and E1B coding sequences, and said cells characterized in that said E1A and at least one of the E1B sequences are separated by at least 4 kb, preferably at least 10 kb, more preferably at least 34.5 kb in said genome. This means that the genome of such cells lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 4, 10 or 34.5 kb, respectively (see e.g. Fig 22 for a schematic representation). It will be clear that wherever the present invention mentions that two sequences are separated by at least a given distance in a genome of a cell, this requirement is deemed to be fulfilled as well when the two sequences are present on separate chromosomes. Hence, the embodiment where the two sequences are on different chromosomes are expressly included within the meaning 'at least x kb apart' or 'separated by at least x kb' in the genome, as used herein. Such an embodiment can be easily checked by methods known to the person skilled in the art, for example by fluorescent in situ hybridization (FISH), which can be used to locate the chromosome and/or chromosome location where a given sequence is present.

The cells of the invention comprise adenovirus sequences in their genome, and preferably said adenovirus sequences encode all E1 proteins but lack sequences encoding pIX, or a part thereof (i.e. such cells preferably do not contain sequences from the open reading frame of pIX (for cells with Ad5 E1 sequences, this means that they preferably not contain nt 3609-4031 of wt Ad5 or parts thereof), and more preferably they also have deletions in the pIX promoter (for cells with Ad5 E1 sequence, preferably no sequences of the pIX promoter downstream from nt 3550, more preferably 3525), in order to prevent significant overlap with adenovirus vectors having pIX under control of its own promoter. The absence of pIX sequences from the genome of the packaging cells aids in preventing overlap between said genome and a recombinant adenovirus vector (US patent 5,994,128).

The cells according to the invention may be derived from immortalized cells such as A549, Hela and the like, in which case a selection marker is required to establish the cells. Preferably, the cells according to the invention are derived from primary cells, in which case selection is provided by the E1A and E1B transforming activity (see examples). In preferred aspects, said cells are derived from retina cells. They may be cells of any origin, including of human origin. For the propagation of human adenovirus, cells of human origin are preferred. The cells may for instance also be of bovine origin for the propagation of recombinant bovine adenovirus (US patent 6,379,944). The origin of the cells may be chosen by the person skilled in the art to be compatible with the recombinant adenovirus of choice.

In one aspect, the cells are derived from primary human retina cells (also referred to as HER cells). Immortalization of such cells with adenoviral E1 sequences has for instance been

described in US patent 5,994,128, in Byrd et al, 1982, 1988, and Gallimore et al, 1986. Primary HER cells can be isolated from fetuses (Byrd et al, 1982, 1988). In other embodiments, the cells are derived from primary human embryonic kidney cells (see e.g. Graham et al, 1977). In yet other embodiments, the cells are derived from primary amniocytes, such as human primary amniocytes (see e.g. US patent 6,558,948 for methods of immortalizing primary amniocytes with adenovirus E1 sequences).

Generation of cells.

The cells according to the invention can be generated by introducing adenovirus E1A and E1B-55K and E1B-19K coding sequences into a precursor or ancestor cell (hence the cells are derived from the precursor or ancestor cell by introduction of the E1 sequences therein). According to the invention, said E1A and at least one of the E1B coding sequences are to be introduced into said precursor cells in an unlinked manner. 'Unlinked' as used in this connotation, is meant to refer to a configuration that is different from the natural configuration of E1A and E1B as found in an adenovirus, i.e. directly adjacent to each other (see e.g. Fig. 14 where the natural -i.e. 'linked'- configuration of E1A and E1B is shown as present in plasmid pIG.E1A.E1B). It is to be noted that prior to the present invention, the person skilled in the art that wished to obtain a packaging cell comprising in its genome adenovirus E1A and both E1B coding sequences, would not contemplate introducing E1A and at least one E1B sequence in an unlinked manner, because: a) it is more work and hence less convenient, and b) both E1A and E1B sequences are required for immortalization. Previous studies, that were done to gain insight into the transforming

capabilities of E1A and E1B proteins, have demonstrated that introduction of E1A and at least one E1B coding sequence can lead to established clones (van den Elsen *et al.*, 1982; Gallimore *et al.*, 1986; Jochemsen et al., 1987; Rao *et al.*, 1992; Nevels *et al.*, 2001). However, the simultaneous introduction of these plasmids, and/or the overlap between the introduced sequences, e.g. in the plasmid sequences, the regulatory sequences such as promoter and/or polyadenylation signal, and the E1B-55K and E1B-19K overlapping sequence likely results in the co-integration of E1A and both E1B coding regions in the same locus in the genome. Indeed such co-integration was observed by van den Elsen *et al* (1982). In one case (Jochemsen *et al.*, 1987) baby rat kidney (BRK) cells were first transfected with E1A and picked clones were then transfected with an E1B plasmid to study the influence of E1B expression on E1A expression. Obviously, this study was done on a specific type of cells and for a reason unrelated to the invention described in this application. Prior to the present invention, there was no reason to separately transfect E1A and at least one of the E1B coding sequences, for the purpose of generating a packaging cell. The formation of HDEP in the absence of substantial overlap between the genome of the recombinant adenovirus and the packaging cell was hitherto not described. Furthermore, the person skilled in the art would in fact would be dissuaded to use this method for obtaining other than BRK-derived cells such as for instance human cells, since Gallimore *et al.* (1986) had described that human cells were only rarely morphologically transformed by expressing E1A only and most clones died in situ or immediately after isolation. The sole case where an Ad12 (subgroup A) E1A expressing plasmid resulted in a clone was identified 114 days post transfection and could only be picked after the clone had

overgrown the dish. Clearly, the person skilled in the art would not be motivated to introduce E1A and at least one E1B gene into precursor cells on a separate moment in time based on the prior art. It is the merit of the present invention to provide such methods for obtaining cells that have E1A and at least one E1B coding sequence separated in their genome. In certain aspects, the cells of the present invention are preferably not baby rat kidney (BRK) cells. In one preferred aspect the cells of the invention are human cells. In a preferred aspect, the cells are derived from HER cells. It will be clear that the cells wherein the E1 sequences are introduced according to the present invention, before said introducing preferably are free from adenovirus E1A and E1B coding sequences in their genome, and only upon introduction of the sequences will obtain the E1 sequences in their genome, thereby becoming immortalized and capable of packaging recombinant adenovirus with deletions in the E1 region. For instance, WO 03/031633 describes the introduction of Ad11 E1B-55k into 293 cells: this will clearly not lead to the advantages of the present invention, since the E1 sequences as already present in 293 remain in the original configuration and will have a E1B ORF less than 4 kb apart from the E1A ORF. In contrast, the present invention provides methods for generating cells having adenovirus E1A and E1B-19K and E1B-55K coding sequences in their genome, as well as the resulting cells from the methods, whereby the nucleic acid sequences comprising E1A and at least one of the E1B coding sequences are sufficiently separated from each other to prevent the simultaneous introduction of all E1A and E1B sequences from the genome of the cells into the genome of a single adenovirus particle.

According to a first embodiment for generating the cells of the present invention, the E1A and at least one of the E1B coding sequences are separately introduced into said precursor cell, by placing them on separate molecules that are introduced into said cell on a different moment in time, and said precursor cells are not BRK cells. In one embodiment hereof, first the E1A and E1B-21K coding sequences are introduced, while the E1B-55K coding sequence is introduced at a later moment in time.

According to a second embodiment for generating the cells of the present invention, at least two nucleic acid molecules together comprising said E1A and said E1B coding sequences are introduced into said precursor cell, characterized in that said at least two nucleic acid molecules lack substantial overlapping sequences. It is believed that the presence of such overlapping sequences would enhance the possibility of at least some way of interaction between said at least two molecules and the subsequent co-integration of the sequences on said molecules into the same locus of the genome of the precursor cell into which said molecules are introduced, whereas it is an object of the present invention to circumvent this. In one embodiment hereof, at least on of the E1A and E1B coding regions are placed under control of different heterologous regulatory sequences, such as promoters and polyadenylation sequences. In another embodiment hereof, the E1B-55K and E1B-19K sequences, which are overlapping, are separated and all overlap between these coding sequences is removed by genetic engineering using knowledge of the redundancy of the genetic code, according to methods known to the person skilled in the art, thereby allowing the introduction of these E1B coding sequences independently and

unlinked from each other. Introduction of the E1A and E1B coding regions in this embodiment may be at the same time for instance by co-transfection of said at least two molecules, but obviously, the first and second embodiments may also be combined, i.e. introduction of at least two molecules comprising nucleic acid sequences together comprising E1A and both E1B coding sequences, wherein said at least two molecules lack substantial overlap with respect to each other, and wherein said at least two molecules are introduced into a precursor cell on a different moment in time.

In a third embodiment, the sequences encoding E1A and at least one of the E1B coding sequences are unlinked by placing them on a single nucleic acid molecule, wherein said E1A and at least one of the E1B coding sequences (obviously this may as well be both E1B coding sequences) are separated by at least 4 kb, preferably at least 6 kb, more preferably at least 8 kb, still more preferably at least 10 kb, still more preferably at least 15 kb, most preferably at least 34.5 kb of nucleic acid, herein referred to as 'spacer' or 'stuffer' nucleic acid. The spacer nucleic acid sequence may advantageously have the characteristics as described infra for spacer nucleic acid sequences, in particular said sequence lacks E1A and E1B coding sequences, may be built up at least in part of intron sequences, and may comprise regulatory sequences of the E1A and/or E1B coding sequence. It will be immediately clear to the skilled person that in an equivalent variant of the third embodiment, said E1A and at least one of the E1B coding sequences may be present on different nucleic acid molecules instead of on one molecule (see e.g. Fig. 13 I, II, and for instance example 3), as long as the sequences are still separated by at least the indicated distance when said different nucleic acid molecules would form a single molecule,

e.g. by (homologous) recombination or ligation or end-to-end joining. The idea is that also in such a case the E1A and at least one E1B coding sequence will still be separated by at least 4 kb, preferably at least 6 kb, more preferably at least 8 kb, still more preferably at least 10 kb, still more preferably at least 15 kb, most preferably at least 34.5 kb, when integrating into the genome of the precursor cell (see e.g. example 3 and Fig. 21). In other words, the third embodiment of the invention can therefore be performed by a introducing into a precursor cell a single nucleic acid molecule ('molecule A') comprising the E1A and E1B-19K and E1B-55K coding sequences, at least two of these three coding sequences being separated by at least 4 kb (embodiment 3a); or equally well by introducing two or more nucleic acid molecules that when these would form a single nucleic acid molecule would form 'molecule A' (embodiment 3b). In certain aspects of the third embodiment, there are no substantially overlapping sequences between the spacer nucleic acids flanking E1A and E1B, nor in the regulatory sequences for the E1A and E1B coding sequences, in order to reduce the chance of interaction and possible homologous recombination between these separated sequences.

Recombinant molecules encoding E1A and E1B.

In one aspect, the invention provides a recombinant molecule comprising nucleic acid sequences encoding the adenoviral E1A proteins and at least one adenoviral E1B protein, characterized in that said nucleic acid sequence encoding E1A proteins and said nucleic acid sequence encoding at least one E1B protein are separated by at least 4 kb, preferably at least 10 kb, more preferably at least 34.5 kb. Such a molecule may be used in the method according to the invention to

generate the cells according to the invention. Such a molecule may take various forms known to the person skilled in the art, such as a plasmid, cosmid, bacterial artificial chromosome, YAC, and the like.

In an equivalent embodiment, it is also possible to have said E1A and E1B protein encoding nucleic acid initially present as separate molecules. Such molecules may optionally be capable of forming a single molecule by homologous recombination, ligation, site-specific recombination, end-to-end joining, and the like. It is therefore an aspect of the invention to provide a set of at least two nucleic acid molecules comprising: a) a nucleic acid molecule encoding the E1A proteins of an adenovirus, wherein said nucleic acid molecule has at least 5kb, preferably at least 17 kb of spacer nucleic acid sequence on both sides of said E1A coding sequence, said spacer nucleic acid not encoding an E1B protein; and b) a nucleic acid molecule encoding an E1B protein of an adenovirus, wherein said nucleic acid molecule has at least 5 kb, preferably at least 17 kb of spacer nucleic acid sequence on both sides of said E1B coding sequence, said spacer nucleic acid not encoding an E1A protein. Said spacer nucleic acid may be any other nucleic acid, and preferably is chosen such that it is inert, i.e. does not contain coding sequences, preferably no known regulatory elements, no highly repeated regions that may lead to chromosomal instability, and the like. Preferably, said spacer nucleic acid sequence flanking E1A encoding sequences does not contain substantial homology with said spacer nucleic acid sequence flanking an E1B protein encoding sequence. The spacer fragment may include regulatory sequences of the E1A or E1B expression cassettes, such as a heterologous promoter and/or polyadenylation site.

The propagation of the recombinant molecules in a host can usually conveniently be performed when the molecules are in circular form. In certain aspects, the recombinant molecules of the invention are in a linear form. This may aid in the transfection of the precursor cell lines, which is generally more efficient when linear molecules are used. Linearization may for instance be effected by digestion of the recombinant molecule with one or more convenient restriction enzymes, as known to the person skilled in the art.

Cell lines lacking E1 sequences in inverted repeat orientation.

Whatever the mechanism of generating HDEP in the absence of substantial overlap between packaging cell and recombinant adenovirus, it is likely that the first step in this process is an integration event of E1 sequences from the genome of the packaging cell into the virus genome. To accommodate for these extra sequences, the virus must subsequently delete adenovirus sequences (Murakami et al, 2002). This step may be more efficient when inverted repeats are present (Steinwaerder et al, 1999). The PER.C6® cell line contains in its genome several repeats of the pIG.E1A.E1B plasmid that was used for the generation of the cell line, some of which repeats are in inverted orientation with respect to each other. Hence, the presence of inverted repeats of the E1 region in the genome of PER.C6® cells may influence the frequency of generating HDEP. It should be noted that the formation of HDEP particles could also occur in other adenovirus packaging cell lines but in such cell lines goes undetected due to the appearance of classical RCA. The absence of E1 sequences in inverted repeat orientation in packaging cell lines will likely result in a lower frequency or even complete absence of generation of HDEP

when recombinant adenovirus is propagated on such packaging cells.

When new cell lines comprising E1 regions in their genome are generated or chosen, it may therefore be desirable to select clones that lack inverted repeats, but rather have only direct repeats, or even a single integration of E1 sequences. It is therefore an aspect of the invention to provide cells, as well as a method for providing or generating cells comprising adenoviral E1 sequences, characterized in that said method includes a step of selecting cells lacking inverted repeats comprising said E1 sequences. The invention provides a cell comprising adenovirus E1 sequences in its genome, wherein said E1 sequences include at least one functional copy of the E1A and E1B-19K and E1B-55K coding sequences, characterized in that said E1 sequences or part thereof are not present in the form of inverted repeats in said genome. In this embodiment, said cell is not a 293 cell or a derivative thereof. If inverted repeats are present, preferably such inverted repeats are not present within 10 kb in said cell, and for the present invention inverted repeats with at least 10 kb of non-E1 intervening sequence are not further considered inverted repeats. The rationale is that when one copy of the E1 sequence is integrated somewhere on a chromosome, and an inverted copy would be integrated on the same chromosome but at a distance large enough to prevent uptake of the whole segment of DNA comprising both repeats in an adenovirus particle, no problem is envisaged. Also when the distance is such that uptake of the fragment is possible (i.e. 10 kb) the duplication of the left end resulting from the inverted repeat sequence gives a virus genome that is too large to be packaged (exemplified in Fig. 13). The site of insertion of E1 sequences in the virus genome is important for the final total

length of the HDEP. It is obvious that the chance that incorporation of an E1-containing genomic fragment results in a packagable genome increases when the insertion is more to the left end of the virus. If the site of insertion is just 3' from the minimal packaging signal then the insert can be as large as 18.5 kb (including 1 copy of E1A and E1B and a inverted repeat sequence) and still remain packagable (assuming a left to right end duplication including 350 bp ITR and packaging signal). This does not mean that an insert of e.g. 17 kb will easily generate HDEPs since the possible sites of integration of such a fragment that still results in a virus of packagable size is limited to 1.5 kb just 3' to the minimal packaging sequence. Thus, the frequency of generation of HDEP will decrease with increasing distance between the E1A and E1B coding regions and may very well still be below detection when that distance is much smaller then the mentioned 18.5 kb.

The same reasoning holds true for direct insertion of E1A and E1B in the absence of inverted repeat sequences. In that case, the total insertion in the genome of an adenovirus cannot be larger than 37.5 kb (including about 3 kb for the E1A and E1B region), but only when it inserts directly 3' from the minimal packaging signal. Therefore, when E1A and at least one of the E1B coding sequences are separated by at least 34.5 kb in the genome of the packaging cell, insertion of both of these sequences into a recombinant adenovirus is prevented. Furthermore, the whole adenovirus genome should then be deleted in a subsequent step, which process may further be much less efficient in the absence of inverted repeat sequences (Steinwaerder et al, 1999). In any case, irrespective of the presence of inverted E1 sequences in the genome, the chance of generating particles containing E1A and

E1B sequences is reduced when the E1A and at least one of the E1B coding sequences are further apart in the genome. Hence, in certain embodiments, said E1A and at least one of E1B coding sequences are separated by at least 4 kb, 6 kb, 8 kb, 10 kb, 12 kb in the genome of the cells according to the present invention. In these embodiments, preferably the E1 sequences are not present in the form of inverted repeats. Preferably, said sequences are separated by at least 15 kb and no inverted E1 sequences are present, which will ensure that the theoretical possibility of generating HDEP in such a cell line by introduction of E1 sequences from the genome of said cell line into an adenovirus is practically reduced to zero (see Fig. 13). In other embodiments, said E1A and at least one of the E1B coding sequences are separated by at least 18 kb, 20 kb, 25 kb, 30 kb. Most preferably, said sequences are separated by at least 34.5 kb. This will ensure that no single integration of both E1A and E1B derived from the genome of the cell can result in the generation of HDEP. Obviously, as indicated above, in certain equivalent embodiments, said E1A and at least one E1B coding sequence are present on different chromosomes of the genome of the cell.

Methods for screening generated clones for the presence of inverted repeats of the E1 sequences are known to the person skilled in the art, and may include PCR, Southern blotting, restriction enzyme analysis, Fiber-FISH, and the like. Upon generating cell clones using a plasmid containing the E1 genes, such as the pIG.E1A.E1B plasmid (US patent 5,994,128), clones can be first established, and in a screening round, those clones lacking said inverted repeat are picked for further use. The cells from the picked clones can then be suitably used for the generation of recombinant adenovirus, and it is expected that the frequency of generating HDEP on

these cells will be significantly lower (or even absent) than in cells that do contain said inverted repeats. It is therefore another aspect of the present invention to provide a cell comprising adenovirus E1A and E1B coding sequences in its genome, characterized in that said E1 sequences are not present in the form of inverted repeats in said genome. For this aspect of the present invention, sequences that are separated by at least 10 kb of non-E1 sequence, are not to be considered as inverted repeats. In a specific aspect, said E1A and E1B sequences are present in said genome in at least two copies per genome. In one aspect, said at least two copies comprise at least two copies present on one chromosome. In another embodiment, said cells comprise in their genome only one copy of the E1A and E1B coding regions, while lacking sequences encoding adenovirus pIX. Of course, a cell comprising only one copy of said E1 sequences in its genome should be expected to have a lower frequency (or even absence) of HDEP generation.

Use of the cells

In another aspect, the invention provides a method for generating recombinant adenovirus in the cells of the invention. This will generate recombinant adenovirus batches having a significantly reduced frequency of HDEP compared to the batches produced on the systems known in the art, preferably no HDEP at all. In particularly preferred embodiments, the vector and packaging cell used for the generation of said recombinant adenovirus lack substantial sequence overlap (i.e. preferably less than 10 nt, or more preferably no overlap at all), and preferably have no overlap, thereby minimizing the chance of homologous recombination between vector and sequences in the packaging cell, resulting

in virus batches having no E1-containing particles (HDEP, RCA).

It is to be noted that the new cells provided according to the invention may also be used for the production of recombinant proteins as described earlier (WO 00/63403), and for producing other (non-adenoviral) viruses as described earlier (WO 01/38362).

It will be clear to the person skilled in the art that the serotype or the nature of the transgene is not critical to the invention. It will for instance be immediately clear that the adenoviral E1 coding sequences that are used for the generation of the cells can be taken from any convenient serotype compatible with the adenovirus that is to be propagated on the cells, such as Ad5, Ad35, Ad11, Ad16, Ad49 etc, or combinations thereof, i.e. E1A from one serotype and at least one of the E1B coding sequences from another serotype (see e.g. WO 02/40665). It will also be clear that in many other aspects the invention may be varied without departing from the scope or the spirit thereof. The invention will now be illustrated by the following examples, which should not be construed to limit the scope thereof.

EXAMPLES

The practice of this invention will employ, unless otherwise indicated, conventional techniques of molecular biology, cell biology, and recombinant DNA, which are within the skill of the art. See e.g. Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, 1989; Current Protocols in Molecular Biology, Ausubel FM, et al, eds, 1987; the series Methods in Enzymology (Academic Press, Inc.); PCR2: A Practical Approach, MacPherson MJ, Hams BD, Taylor GR, eds,

1995.

Example 1. *Generation of complementing cell lines using separate nucleic acids encoding E1A and E1B proteins*

The complete morphological transformation of primary cells by adenovirus E1 genes is the result of the combined activities of the proteins encoded by the E1A and E1B regions. The roles of the different E1 proteins in lytic infection and in transformation have been studied extensively (reviewed in Zantema and van der Eb, 1995; White, 1995, 1996). The adenovirus E1A proteins are essential for transformation of primary cells. The concomitant induction of apoptosis is counteracted by both E1B-19K and E1B-55K, although by different mechanisms. Although the E1A region encodes several proteins, the whole region is usually referred to as the E1A coding region, and in all embodiments the 'E1A coding sequence' as used herein refers to the sequences encoding all E1A proteins.

In rodent cells, the activity of E1A together with either E1B-19K or 55K is sufficient for full transformation although expression of both E1B proteins together is twice as efficient (Gallimore *et al.*, 1985; Rao *et al.*, 1992). In human cells however, the activity of the E1B-55K protein seems to be more important given the observation that E1B-55K is indispensable for the establishment of an immortal transformed cell line (Gallimore *et al.*, 1986). In adenovirus infection and virus propagation the E1A proteins function in activation of the adenovirus genes including E1B and other early regions probably via interaction with the TATA-binding protein (reviewed in Zantema and van der Eb, 1995). E1B-55K expression is important in the late phase of infection for shut-off of host protein synthesis and selective transport from the

nucleus to the cytoplasm of viral-encoded proteins (Babiss *et al.*, 1985; Pilder *et al.*, 1986). Adenoviruses that are deleted for E1B-55K show decreased replication on non-complementing human cell lines (Harada and Berk, 1999). Thus the E1A- and E1B-55K-encoded proteins are necessary for transformation of primary human cells and for efficient virus replication in human cells.

Non-homologous recombination can result in incorporation of E1 sequences from the cellular genome of the packaging cell into the recombinant adenovirus. The helper-dependent E1-containing particles that finally result from the initial recombined adenovirus are able to complement replication of the replication-deficient vector on non-complementing (human) cells but are not capable of autonomous replication. This complementation is mediated by both E1A and E1B-55K and possibly E1B-19K functions. Thus, if the chance that both E1A and E1B-55K (and preferably E1B-19K) functions end up in the adenoviral vector is eliminated or reduced, then the formation of HDEP will be eliminated or reduced.

Here we describe examples of functional plasmids expressing either E1A, E1A and E1B-19K, E1B (E1B-19K + E1B-55K) or E1B-55K that are used to generate adenoviral packaging cell lines with E1A and E1B-55k regions separated from each other.

Construct pIG.E1A.E1B (Fig. 14; SEQ.ID.NO. 1), containing the Ad5-E1 region (nucl. 459-3510 of the Ad5 genome (Genbank Acc. No. M73260) operatively linked to the human phosphoglycerate kinase (PGK) promoter and hepatitis B virus poly-adenylation sequence, has been described previously (US patent 5,994,128).

Generation of construct pIG.E1A

Construct pIG.E1A was made by digestion of pIG.E1A.E1B with HincII followed by purification of the resulting 5 kb fragment from gel using QIAEX II gel extraction kit (Qiagen) according to manufacturers instructions. Religation of the isolated fragment and transformation into STBL2 competent cells (Invitrogen) gave pIG.E1A (Fig. 1). This construct contains nt. 459 to 1578 from the Ad5 genome (Genbank Acc. No. M73260)

Generation of construct pIG.E1AB21

Construct pIG.E1A was digested with XbaI and HpaI and the resulting 4.8 kb fragment was isolated from gel as above. Construct pIG.E1A.E1B was digested with BsrGI and treated with Klenow enzyme (New England Biolabs) to blunt the 5′ protruding ends. DNA was then purified with the QIAquick PCR purification kit (Qiagen) according to manufacturers instructions and subsequently digested with XbaI. The resulting 913 bp fragment containing the 3′ part of E1A and the E1B19K coding sequence was isolated from gel as described. Ligation of the two isolated fragments and transformation into DH5α-T1^{r} cells (Invitrogen) gave construct pIG.E1AB21 (Fig. 2). This construct thus contains nt. 459 to 2253 from the Ad5 genome (Genbank Acc. No. M73260) whereby the E1A sequence is driven by the PGK promoter and the E1B promoter drives the E1B-19K gene.

The Ad5 E1B-19K gene is sometimes also referred to as the Ad5 E1B-21K gene, because the predicted amino acid sequence constitutes a 20.6 KD protein (for instance, several of the plasmids and primers in this application have 21K as part of their names).

Generation of construct pCR5B

A construct containing the Ad5-E1B region was then generated as follows. First a PCR fragment was generated with primers 5E1Bfor-1: 5′- CGG AAT TCG GCG TGT TAA ATG GGG CG-3′ (SEQ.ID.NO. 2) and 5E1B-rev: 5′- TAG CAG GCG ATT CTT GTG TC-3′ (SEQ.ID.NO. 3), using pIG.E1A.E1B DNA as template and Pwo DNA polymerase (Roche) according to manufacturers instructions with DMSO at 3% final concentration. The amplification program was 94°C for 2 minutes followed by 30 cycles of (94°C for 30 seconds, 50 °C for 30 seconds and 72 °C for 1 minute) and ended by 72 °C for 10 minutes. The resulting 481 bp amplified fragment was purified with the QIAquick PCR purification kit (Qiagen) and ligated to the pCR-ScriptAmp vector from the PCR cloning kit (Stratagene) in the presence of SrfI enzyme according to manufacturers instructions. The (blunt) ligation gave two orientations of the insert in the vector of which the one with the largest fragment between the EcoRI site in the (5′) end of the insert and the EcoRI site in the vector was arbitrarily chosen. This resulted in construct pCR5B. Correct amplification of the target sequence (between KpnI and EcoRI sites) was verified by sequencing.

Generation of construct pE1B

pCR5B was digested with KpnI and EcoRI and the 415 bp fragment was isolated from gel using the QIAquick gel extraction kit (Qiagen). Construct pIG.E1A.E1B was then also digested with EcoRI and KpnI and the resulting 5.2 kb vector fragment was isolated from gel as above. Ligation of the isolated fragments and transformation into DH5α-T1^{r} cells (Invitrogen) resulted in construct pE1B (Fig. 3). The E1B sequence in this construct constitutes nt. 1642 to 3510 from the Ad5 genome.

The transforming ability of the new constructs in comparison with the full length Ad5E1 expression construct was then tested. Hereto, primary human embryonic retina (HER) cells were isolated (see e.g. Byrd et al, 1982, 1988) and seeded in 6 cm dishes in DMEM medium (Gibco BRL) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS, Gibco BRL). At 60-70% confluency cells were transfected with in total 20 μg DNA/ dish using the $CaPO_4$ co-precipitation kit (Invitrogen) according to manufacturers instructions. Two weeks later transformed clones were visible as foci in a monolayer of primary cells. Cells in a focus showed a clearly different morphology compared with the primary cells. Table II depicts the amounts of transformed clones that were obtained with each of the transfections.

The results confirm previous observations that primary cells can be transformed with Ad5-E1A and E1B19K genes (pIG.E1AB21). It should be noted that these foci were generally smaller than the ones obtained with the transfection where the complete E1B region was present. Also, after picking the foci resulting from transfections with construct pIG.E1AB21 and seeding into 96-well plates, no sustained cell growth was obtained and all cells died. In all other cases most of the picked foci resulted in viable cell clones.

Since transfection with pIG.E1AB21 initially resulted in transformed cells, it is possible to re-transfect the cells with an E1B-55K expression construct, e.g. 5-10 days following the first transfection. This ensures that cells are obtained that have incorporated both expression cassettes on different loci in the genome.

This experiment further confirms that it is possible to generate and establish transformed cell clones by using two separate plasmids for the E1A and E1B genes (pIG.E1A + pE1B).

However, because both plasmids were transfected together and contain considerable sequence overlap (plasmid backbone and promoter/polyA), it is possible that integration of E1A and E1B took place in the same locus in the genome.

This may be avoided using fragments with expression cassettes only (no vector sequences) and having no sequence overlap. The sequence overlap can be removed from regulatory elements, such as the promoters and polyadenylation (polyA) sequences, by using different regulatory sequences for the two expression constructs with the E1A and E1B sequences. Preferably these sequences are sufficiently divergent to prevent overlap that could lead to the formation of paired structures found during a homologous recombination process. Any promoter and polyA sequence can be used. Preferably the regulatory sequences are different from those from the transgenes in the recombinant adenovirus that will be propagated on these cells. Obviously this can only be determined when a given recombinant adenovirus is propagated on these cells at a later stage and will depend on the particular recombinant adenovirus. Hence it is convenient to choose the regulatory sequence of such transgenes later, such that they differ from those of the E1A and E1B sequences in the cells that are established by the present invention. However, since many currently available recombinant adenovirus vectors carry transgenes regulated by a CMV promoter and an SV40 polyA sequence, the preferred regulatory sequences for the E1A and E1B constructs as exemplified herein are different from the CMV promoter and the SV40 polyA sequence. In view of regulatory issues, further these regulatory sequences are preferably not of viral origin.

To this end, the plasmids described above are further modified as described below.

Generation of construct pCC.E1A and pCC.E1AB21

The E1A sequence was amplified with the following primers: 5E1A-For: 5'- CCG AAT TCG ATC GTG TAG TG-3' (SEQ.ID.NO. 4) and 5E1A-rev: 5'- CGG GAT CCA TTT AAC ACG CCA TGC AAG-3' (SEQ.ID.NO. 5). The reaction was done on pIG.E1A.E1B template DNA using Pwo DNA polymerase (Roche) according manufacturers instructions but with a final concentration of 3% DMSO. The PCR program was set on 94°C for 2 minutes followed by 30 cycles of (94°C for 30 seconds, 58 °C for 30 seconds and 72 °C for 120 seconds) and ended by 72 °C for 8 minutes. The resulting 1.2 kb fragment contains the E1A sequence from Ad5 (nucleotide 459 to 1655 as in Genbank Acc. No. M73260) flanked by EcoRI (5') and BamHI (3') sites.

A second PCR fragment was generated using primer 5E1A-For with reverse primer: 5E1AB21-rev: 5'- CGG GAT CCT CAT TCC CGA GGG TCC AG-3' (SEQ.ID.NO. 6), using the same conditions.

The resulting 1.8 kb fragment contains the E1A and E1B-19K sequence from Ad5 (nucleotide 459 to 2244 as in Genbank Acc. No. M73260) flanked by EcoRI (5') and BamHI (3') sites. Both PCR fragments were isolated from agarose gel, purified with QIAEX II gel extraction kit (Qiagen) and cloned in a PCR cloning vector; pCR-TOPOblunt (Invitrogen) and the sequence was verified. The constructs were then digested with EcoRI and BamHI and the insert fragments were isolated from gel using the QIAEX II gel extraction kit (Qiagen) as above.

Each of the isolated fragments was then ligated into vector pCC101 (see below) that was first digested with EcoRI and BamHI and purified from gel as above. Transformation into electro competent DH10B cells (Invitrogen) generated constructs pCC.E1A (Fig. 4) and pCC.E1AB21 (Fig. 5). A synthetic polyadenylation signal (SPA) is present in these

plasmids (derived from construct pCC271 as described in WO 02/40665).

Generation of pCC101

pCC100 (see below) was digested with XbaI and the resulting linear fragment was purified from gel using the QIAEX II gel extraction kit as above. A linker was prepared by annealing the oligonucleotides X-SM-1: 5′- CTAGGTCGACCAATTG-3′ (SEQ.ID.NO. 7) with X-SM-2: 5′- CTAGCAATTGGTCGAC-3′ (SEQ.ID.NO. 8). Hereto, 1 µg of each oligonucleotide was mixed with 2 µl 10x NEB2 buffer (NEB) and milliQ H_2O to a final volume of 20 µl. The mixture was placed at 98°C and slowly cooled to 4°C in a PCR machine (cooling rate of 2°C / minute). The annealed linker was then ligated to the isolated XbaI digested fragment using a 4x molar excess of linker over fragment. The ligated DNA was purified with the QIAquick PCR purification kit (Qiagen) according to manufacturers instructions and digested with XbaI to remove self-ligated vector DNA. Following heat-inactivation of the XbaI enzyme, the mixture was then used to transform DH5α-T1^r competent cells resulting in pCC101 (Fig. 6).

Generation of pCC100

Construct pCC271 (described in WO 02/40665) was digested with EcoRI and PstI and the 3 kb vector fragment was isolated from gel as described above. A linker was prepared by annealing oligonucleotide EcoPst-3: 5′- AAT TGA TAT CGA ATT CGC CGA GCT CGT AAG CTT GGA TCC CTG CA-3′ (SEQ.ID.NO. 9) with oligonucleotide EcoPst-4: 5′- GGG ATC CAA GCT TAC GAG CTC GGC GAA TTC GAT ATC-3′ (SEQ.ID.NO. 10). Hereto, oligonucleotides were mixed as described above and annealed by incubation at

98°C for 2 minutes, 65°C for 30 minutes and room temperature for 2 hrs. The isolated vector fragment was then ligated to excess annealed oligo and transformed into DH5α-T1^r competent cells resulting in construct pCC100.

Generation of pCC200

Plasmid pBR322 (GenBank J01749.1) was digested with EcoRI and then blunted with Klenow enzyme, followed by purification with QIAquick PCR purification kit (Qiagen). After a second digestion with NheI, the 4150 bp vector fragment was isolated from agarose gel using the QIAEX II gel extraction kit (Qiagen). In parallel, construct pCC100 was digested with BsaAI, blunted with Klenow enzyme and purified with the QIAquick PCR purification kit (Qiagen), followed by a second digestion with XbaI and isolation of the 350bp fragment from agarose gel. The fragments were then ligated and transformed into chemical competent STBL-2 cells (Invitrogen), resulting in pCC200 (Fig. 15).

Generation of pCC105

Construct pCC105 contains the human PGK promoter and a poly-adenylation sequence derived from the human COL1A2 gene. First, the COL1A2 poly-adenylation sequence (Natalizio *et al.*, 2002) was amplified by PCR from human genomic DNA as described by the authors using recombinant Taq polymerase (Invitrogen) and primers COL1A2F: 5′- CAG CTA GCC TGC AGG AAG TAT GCA GAT TAT TTG -3′ (SEQ.ID.NO. 11) and COL1A2R-sal: 5′- ACA CGT CGA CGG CTG GTA GAG ATG C -3′ (SEQ.ID.NO. 12).

Herewith the published sequence is extended at the 5′-end by a SbfI restriction sequence and at the 3′- end by a SalI restriction sequence. The resulting PCR fragment was cloned into pCR-TOPO-TA using the pCR-TOPO4 TA cloning kit

(Invitrogen). After verification of the insert by sequencing, the 277bp insert was isolated from the TOPO vector by digestion with SbfI and SalI, and purified with the QIAquick PCR purification kit (Qiagen). In parallel, the plasmid pCC101 was also digested with SbfI and SalI, followed by gel electrophoresis. The 2965 bp vector fragment was isolated from agarose gel using GeneClean Kit (Bio101) according to manufacturers instructions. This vector fragment was ligated to the purified COL1A2pA fragment in equimolar amounts and transformed to chemical competent STBL-2 cells (Invitrogen). This gave plasmid pCC105 (Fig. 7).

Generation of pCC205

Construct pCC205 was made by digestion of pCC200 with SalI and EcoRI, followed by purification of the 4225 nt vector fragment from agarose gel, using QIAEX II gel extraction kit (Qiagen). In parallel, the 310 nt COL1A2 polyA was isolated from construct pCC105 by digestion with EcoRI and SalI, followed by gel electrophoresis and purification of the fragment using the QIAEX II gel extraction kit. The two fragments were then ligated in equimolar amounts and transformed to DH5α-T1r, resulting in pCC205 (Fig. 16).

Cloning of pCC.55Kcol

Vector pCC205 is used for the construction of a plasmid expressing the Ad5 E1B-55k protein. Hereto, a PCR product is generated using the following primers: 55KforE: 5′-GGA ATT CGC CAC CAT GGA GCG AAG AAA CCC ATC TGA-3′ (SEQ.ID.NO. 13) and 55KrevB: 5′-gga tcc TCA ATC TGT ATC TTC ATC GCT AGA GCC-3′ (SEQ.ID.NO. 14). PCR is performed with Pwo DNA polymerase according to the manufacturers protocol in the presence of 3% DMSO. The amplification is done on pIG.E1A.E1B and the program

is set on 94°C for 2 minutes followed by 30 cycles of (94°C for 30 seconds, 60 °C for 30 seconds and 72 °C for 90 seconds) and ended by 72 °C for 8 minutes. The resulting 1510 bp amplified fragment contains the E1B-55K sequence nt. 2019 to 3510 from the Ad5 sequence. The fragment is purified with the QIAquick PCR purification kit (Qiagen), digested with EcoRI and BamHI followed by gel electrophoresis for the removal of the cleaved ends. This fragment is then purified from agarose gel using the GeneCleanII kit (Bio101) and ligated to pCC205, which is also digested with EcoRI and BamHI and isolated from agarose gel as described above. The ligation mixture is transformed to chemical competent STBL2 cells (Invitrogen) resulting in construct pCC.55Kcol (Fig. 8).

Cloning of pIG.E1B

A PCR fragment was generated with Pwo DNA polymerase (Roche) according to manufacturer's instruction in the presence of 3% DMSO. The following primers were used in the amplification reaction: 5E1Bstart: 5'-GGA ATT CCT CAT GGA GGC TTG GG-3' (SEQ.ID.NO. 15) and 5E1Brev2: 5'-GTG TCT CAC AAC CGC TCT C -3' (SEQ.ID.NO. 16). The amplification was done on pIG.E1A.E1B and the program was set on 94°C for 2 minutes followed by 5 cycles of (94°C for 30 seconds, 56 °C for 30 seconds and 72 °C for 60 seconds) these cycles were then followed by another 35 cycles (94°C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds) and ended by 68 °C for 8 minutes. The 390 nt PCR fragment was then digested with KpnI and EcoRI resulting in a 347 nt fragment that was isolated from agarose gel using the QIAEX II Gel Extraction Kit (Qiagen).

This fragment was then ligated to the 5713 bp pIG.E1A.E1B vector fragment resulting from digestion with KpnI and partially with EcoRI, subsequent isolation from gel and

purified with the QIAEX II gel extraction kit (Qiagen). After ligation, the mixture was transformed into chemically competent STBL-2 cells, resulting in the plasmid pIG.E1B (Fig. 9). pIG.E1B contains nt. 2019 to 3510 from the Ad5 genome.

Cloning of pCC.E1Bcol

For the construction of a plasmid carrying both the 19K and 55K Ad5 E1B coding sequences, the plasmid pCC.55Kcol is digested with EcoRI and KpnI. The 5970nt vector fragment is isolated by gel electrophoresis and purified from agarose gel with the GeneClean kitII (Bio101) as above. Construct pIG.E1B is then also digested with KpnI and EcoRI and the 347 nt fragment is isolated from gel and purified with the QIAquick gel extraction kit (Qiagen). Ligation of this insert and the isolated pCC.55Kcol vector fragment and transformation into STBL2 cells gives construct pCC.E1Bcol (Fig. 10). This construct contains nt. 1711 to 3510 from the Ad5 genome sequence.

Cloning of pEC.E1B

The Human Elongation Factor 1-α promoter (EF1-α) is isolated from plasmid pEF/myc/nuc (Invitrogen) by digestion with EcoRI and PmlI. After digestion the fragment is blunted with Klenow enzyme and the 1183 nt EF1-α promoter fragment is then isolated by gel electrophoresis and purified with the GeneCleanII kit (Bio101). In parallel, the vector pCC.E1Bcol is digested with BstXI, followed by T4 DNA polymerase treatment to make the ends blunt and purified over a PCR purification column (Qiagen). Then a second digestion is performed with EcoRV, followed by gel electrophoresis. The 5827 nt vector fragment is then purified with the GeneCleanII

kit (Bio101). Both the EF1-α fragment and vector fragment are ligated together in an equimolar amount and transformed to chemical competent STBL-2 cells (invitrogen), which results in the plasmid pEC.E1B (Fig. 11) that contains the same Ad5-E1B sequence as in plasmid pCC.E1Bcol.

Cloning of pSC.55K

The SV40 promoter was amplified from pEF/myc/nuc plasmid DNA (Invitrogen) by using recombinant Taq DNA polymerase (Invitrogen) and the following primers: SV40.forS: 5'-CAA CTA GTA CAT GTG GAA TGT GTG TCA GTT AGG-3' (SEQ.ID.NO. 17) and SV40.RevERI: 5'-GGA ATT CAG CTT TTT GCA AAA GCC TAG G-3' (SEQ.ID.NO. 18). The amplification program was set at 94°C for 2 minutes followed by 5 cycles of (94°C for 30 seconds, 48 °C for 30 seconds and 72 °C for 45 seconds) then 25 additional cycles of (94°C for 30 seconds, 58 °C for 30 seconds and 72 °C for 45 seconds) and ended by 68 °C for 8 minutes. The resulting 357 bp amplified fragment (nucleotide 266 to -71 from SV40 sequence GenBank Acc. No. J02400) was isolated from agarose gel with the QIAEX II gel extraction kit (Qiagen). This fragment was then cloned into pCR-TOPO, using the PCR TOPO4blunt cloning kit (Invitrogen). After sequence verification, the 357bp insert was isolated from the TOPO vector by digestion with EcoRI and SpeI, and isolated from agarose gel with the QIAEX II gel extraction kit (Qiagen). In parallel, the plasmid pCC.55Kcol (which has a pBR322-backbone) is digested with AvrII and EcoRI, followed by gel electrophoresis. The 5508 nt vector fragment is isolated from gel as described above and ligated in equimolar amount to the digested PCR fragment. The ligation mixture is transformed into chemical competent STBL-2 cells resulting in the plasmid

pSC.55K (Fig. 12) that contains the same Ad5 E1B-55K sequence as in pCC.55Kcol.

To generate transformed clones from primary HER cells, DNA fragments containing the appropriate expression cassettes are isolated from gel. The elimination of (overlapping) vector sequences is believed to reduce the chance of co-integration. Hereto, pCC.E1A and pCC.E1AB21 are digested with BsaAI and AflIII and the insert fragments are purified from gel using an ELU-Trap apparatus (Schleier and Schuell) according to manufacturers instructions. This apparatus enables isolation of large amounts of DNA fragments. Constructs pEC.E1B and pSC.55K are digested with AatII/HincII and AflIII/HincII respectively and insert fragments are isolated as above. Primary HER cells are cultured and transfected as described above. Fragments isolated from pCC.E1A and pEC.E1B are combined for transfection. The DNA fragments isolated from pCC.E1AB21 are combined with pSC.55K or transfected alone. The latter cultures are then retransfected with the DNA fragment isolated from pSC.55K 7-10 days following the first transfection depending on the size of the observed clones. One day before transfection half of the dishes transfected with E1AB21 alone is passed to 10 cm dishes and then re-transfected, the other half of the dishes is re-transfected without prior passage.

The transformed clones that result from these transfections are picked and further expanded in 96-well plates and subsequent larger formats. The integration site and copy number of the fragments is investigated with Southern blots and by PCR to reveal whether insertions occurred in close vicinity. Clones where E1A and E1B 55K are present in single copy, or present in more than one copy but without inverted

repeat conformation of these sequences in the genome of the transfected cells, or wherein E1A and at least one of the E1B coding sequences (in this case E1B-55K) are separated by at least 4 kb, preferably by at least 10 kb, more preferably by at least 34.5 kb, are preferably used further.

Example 2. *Generation of complementing cell lines using nucleic acids that have E1A and E1B regions separated by large spacer sequences.*

As described in the previous example, it is possible to generate stably transformed cell clones from primary cells using separate plasmids for E1A and E1B. However when DNA fragments are transfected together, there is still a chance that the transfected fragments end up in near proximity of each other although this chance is reduced in the absence of considerable sequence overlap. The possibility of E1A and E1B expression cassette integration with less than 30 kb non-E1 sequence in between in the chromosome can be avoided by flanking the different E1 expression cassettes with large pieces of non-(E1-)coding DNA sequences (so called 'spacer' fragments). If an E1A-carrying fragment integrates next to an E1B-containing fragment in the genome of the generated packaging cell, the large flanking sequences should create enough distance to eliminate the chance that the E1A and E1B encoding sequences recombine into the same recombinant vector molecule upon propagation of a recombinant adenoviral vector in said packaging cell. Non-limiting examples of large spacer DNA fragments that can be used are large sequences derived from e.g. the human dystrophin gene or the human Apo-E1 gene. Other spacing molecules can also be used, even from non-human sources. Preferably, the flanking sequences do not contain coding regions for functional proteins and can thus be derived

from intron sequences. Also the E1A and E1B sequences do not need to be on different molecules but may be situated on the same large molecule, such as a cosmid. Examples of these E1A and E1B carrying molecules are given in Fig. 13. In preferred embodiments, the distance between the E1A and E1B coding regions is >34.5 kb because then co-insertion of the two regions into a virus results in a genome that is too large to be packaged. This situation will for instance be reached when two separate cosmid fragments of approximately 40 kb having the expression cassettes roughly in the middle are used. However, assuming that E1 in the form of inverted repeat structures contributes to the frequency of HDEP generation, then a construct where E1A and E1B cassettes are located on a single cosmid fragment of a total length of approximately 22 kb (like in Fig 13I) suffices even when a second copy would become located in the genome of the cell in inverted orientation next to the first. Full integration of the mirror-imaged structure would then also involve a fragment of >38 kb. Also, if the presence of inverted repeats increases the frequency of HDEP formation, then a situation where multiple copies are integrated as direct repeats is considered less of a problem even when the DNA fragment that includes 2 full copies of E1A and E1B has a length of $\leq$ 20 kb. In the absence of inverted repeats, integration of any E1-containing fragment larger than the space left in the recombinant virus (i.e. 38 kb minus actual genome length of recombinant virus) into the recombinant virus forces it to delete parts of the essential viral sequences as a sequential step to ensure packaging and thus propagation. Analysis of the first HDEP genome that arose via homologous recombination (Murakami et al., 2002) showed that deletion of viral sequences is possible.

DNA sequences that integrate into the genome of a cell are not always accessible for activating transcription factors due to chromatin-associated repression. Especially when large fragments of intron DNA are used that originate from genomic regions that are normally not active in the cell that will be transduced with these sequences, gene expression may be shut-off due to inactive chromatin. Recently, sequences were identified that can inhibit this repression. Examples are the chicken β-globin HS4 element (US patent 5,610,053) that functions as an insulator sequence, a Drosophila scs or scs' element (Kellum et al, 1991; Farkas et al, 1992), and a series of sequences in the human genome (so-called anti-repressor elements or STAR elements) identified by a specific screen for anti-repressor elements (Kwaks *et al.*, 2003; WO 03/004704). Incorporation of such sequences in the E1A and E1B expression constructs, which sequences prevent positional silencing of the E1A and/or E1B genes, also enhances the amount of immortalised clones. Therefore, in certain embodiments of the present invention, one or more STAR-elements, for instance STAR 7 (Genbank accession number AY190751) or STAR 40 (Genbank accession number AY190756), are present in at least one of the E1A and/or E1B expression constructs. Preferably said elements are flanking both sides of the E1A and/or E1B coding sequences, i.e. the expression constructs may comprise from 5' to 3': STAR element - expression regulatory sequence (e.g. promoter) - E1A coding sequence; or E1A + one E1B coding sequence; or one E1B coding sequence; or both E1B coding sequences - polyadenylation sequence - STAR element.

Example 3 *Generation of cell lines with E1A and E1B on separate constructs and flanked by stuffer DNA*

This example describes the generation of new cell lines according to the invention by co-transfection of a first DNA fragment having an E1A expression cassette flanked by large stuffer DNA and a second DNA fragment having an E1B expression cassette flanked by plasmid DNA.

The human dystrophin intron 44 sequence (Genbank Acc. No. M86524) cloned in a cosmid vector backbone (pdys44; Fig. 17) was taken in this example as a stuffer DNA surrounding the E1A expression plasmid.

Construct pCC.E1A (described in example 1; Fig. 4) was digested with AflIII and AvrII (New England Biolabs) and protruding ends were made blunt with Klenow enzyme (New England Biolabs). Digested fragments were separated on a 0,5% TAE agarose gel and the 2 kb fragment corresponding to the PGK-E1A expression cassette was purified using the gel extraction kit (Qiagen) according to manufacturers description.

The construct pdys44 (Fig 17) was digested with BglII (New England Biolabs) and protruding ends were made blunt with Klenow enzyme. Fragments were separated on a 0,5% TAE agarose gel and the 27 kb fragment containing the backbone plasmid and part of the dystrophin intron was excised. The gel slice was then put in a syringe containing a bit of glass wool and, using the plunger, the buffer containing DNA was pushed in an eppendorf tube. The thus obtained DNA solution contained approximately 5ng/µl and was used directly in the ligation reaction with the purified 2 kb PGK-E1A fragment.

Transformation into DH5αT1 competent cells resulted in construct p44-1.ccE1A (Fig. 18). The construct contains E1A

under control of the human PGK promoter and a synthetic polyA signal.

Construct pE1B (described in example 1; Fig 3) was used in this example as the E1B expression plasmid. The plasmid contains E1B under control of its own promoter and a hepatitis B virus (HBV) polyA signal.

Construct p44-1.ccE1A was digested with XhoI and PmeI and digested DNA was purified by phenol/chloroform (1:1) extraction followed by Ethanol precipitation (resulting in E1A coding sequences flanked by a stuffer of about 11.6 kb upstream (including the PGK promoter) and about 6.5 kb downstream (including the synthetic polyA signal) of the E1A coding sequences). DNA was then pelleted, washed with 70% ethanol and aseptically dissolved in sterile endotoxin-free TE. Plasmid pE1B was digested with ScaI and purified as above (resulting in E1B coding sequences flanked by a stuffer of about 1.4 kb upstream and more than 2.3 kb downstream of the E1B coding sequences, the downstream stuffer including the HBV polyA sequence).

Primary human HER cells were cultured and transfected on passage number 6 (PN6) in one series of transfections and on PN9 in a second series of transfections. HER cell culture and transfections were done according to the method described in Example 1. The E1A and E1B containing DNAs prepared above were mixed in different proportions in separate transfections (both fragments lack significant overlap with respect to each other derived from vector sequences or regulatory sequences, thereby reducing the chances of homologous recombination between the two fragments; if both fragments would form a single nucleic acid molecule (e.g. by end-to-end joining or ligation, a theoretical possibility), and subsequently integrate into the genome as a single unit, the E1A and E1B coding sequences in

the resulting cells would be separated by more than 4 kb of stuffer sequences (at least 6.5 kb (downstream E1A) + 1.4 kb (upstream E1B) = at least 7.9 kb between the E1A and E1B coding sequences, in theory). A total of 8 dishes were transfected with approximate equimolar ratios of both constructs (17 µg E1A and 3 µg E1B plasmid) and 3 dishes were transfected with 10 µg of each fragment. Foci were observed in both series of transfections but in the case of 10 µg of each construct relatively more foci were obtained. Construct pIG.E1A.E1B digested with AseI and BglI was used as a positive control (20 µg DNA/dish) on separate dishes. As expected, the efficiency of foci formation was higher with the (single) positive control plasmid than with the two separate fragments (from p44-1.ccE1A and pE1B). The pIG.E1A.E1B plasmid was approximately 10x more efficient. Still, both with the positive control plasmid and with the two-fragment transfection about 80-90% of the transformed cell clones that were picked were found to be viable and established as a cell line.

These experiments clearly show that it is feasible to transform primary cells by co-transfection with E1A and E1B genes on separate DNA fragments and flanked by (non-overlapping) stuffer DNA. A total of 6 clones HER01-B-71 (deposited on 1 October 2004 at the European Collection of Cell Cultures (ECACC) under number 04100101), HER01-H-87 (deposited on 1 October 2004 at the ECACC under number 04100102), HER01-H-86, HER01-H-88, HER01-H-89 and HER01-B-90 were analysed further.

Expression of the E1 genes was analysed using specific antibodies for the E1 proteins on Western Blots. A total

amount of 10μg protein from a lysate of the generated cell clones was used in the Western blot assay. The samples were denatured for 15 minutes at 70°C, after addition of ¼ volume NuPage sample buffer (Invitrogen). As a positive control, PER.C6® cell lysate was used. Untransfected primary HER cell lysate (passage number 6) served as a negative control. The samples were run on a 10% BisTris SDS page gel (Invitrogen), alongside a Seeblue plus2 prestained marker (Invitrogen). A Western blot was prepared from the gel. The following antibodies were used: either 1) E1A: mouse anti-human Ad2.E1A (1:400, Santa Cruz), or 2) E1B.19K: rat anti-human E1B 21K monoclonal (1:500, Oncogene), or 3) E1B.55K: mouse anti-human 55Kda (harvested from hybridoma cell line C9A1C6, obtained from Dr. R. Hoeben, LUMC, Leiden). The following antibodies were used as a second antibody: 1) E1A: Goat-anti mouse IgG-HRP (Biorad), 2) E1B.19K: Goat-anti rat IgG-HRP (Epcam), 3) E1B.55K: μl Goat-anti mouse IgG-HRP (Biorad). The proteins were visualized by the use of an ECL+ assay (Amersham).
From these experiments it is clear that all tested clones express E1A and E1B proteins and that the levels are comparable to those in PER.C6® cells (Fig. 19). This confirms that the transformation of the primary cells is indeed induced by Ad5 E1 expression and not the result of a spontaneous event.

Functional expression of Ad5 E1 genes in these cell lines can also be tested by showing that the cells are able to complement E1-deleted Ad5 viruses. Therefore, the 6 different cell clones were tested for replication of an Ad5.eGFP vector, an E1-deleted (deletion of nt. 455-3510 of the Ad5 sequence) Ad5-based adenovirus vector expressing Green Fluorescent

Protein. Hereto, cells were seeded at a density of $1x10^6$ cells/well and infected the day after with a multiplicity of infection (MOI) of 5 virus particles (VP)/cell. As a positive control PER.C6® cells were also seeded and infected with an MOI of 5. Five days later full cytopathogenic effect (CPE) was seen in all wells. Cells and medium were harvested, freeze/thawed three times and centrifuged to remove cell debris. Supernatants (crude lysates) were then used to infect A549 cells. Hereto, $5x10^5$ A549 cells were seeded in 24-well plates and one day later infected with 50 µl of the crude lysates. Two days later A549 cells were harvested and analysed for GFP expression by FACS. Results show that all clones are able to complement an Ad5.eGFP vector (Fig 20).
Notably, the adenovirus vector (E1-deleted, lacks nt 455-3510) has no overlap with the E1 sequences present in the cell line (Ad5 nt 459-3510), and therefore the combination of adenovirus vector with the new cell lines in this example amounts to a packaging system according to the invention, and the generation of recombinant adenovirus in this example amounts to a method of generating a batch of recombinant adenovirus according to the invention.

Genomic DNA from the generated cell lines is tested on Southern blots, using E1A and E1B probes, to demonstrate that the E1A and E1B coding sequences are separated by more than 4 kb in the genome of the cells. Hereto, the genomic DNA is digested with the restriction endonucleases *Eco*RV and *Bgl*II (Fig. 21). The digested DNA is size separated by gel electrophoresis (e.g. using field inversed gel electrophoresis, FIGE, to enable separation of large DNA fragments) and then transferred to a nylon membrane by capillary blotting. Three different probes are radioactively

labeled for hybridization of the blots. One Ad5.E1A probe is generated from an *Eco*RV-*Sal*I restriction fragment (1330 bp; see Fig 21) located 3′ from the *Eco*RV site in the Ad5.E1A gene. Two Ad5.E1B probes are generated; Ad5.E1B(5′), from a *Bss*HII-*Bgl*II fragment (1354 bp) and Ad5.E1B(3′), from a *Bgl*II-*Bsr*GI fragment (652 bp), which are located at the 5′ side and the 3′side of the *Bgl*II site in the Ad5.E1B gene respectively. Identical blots containing the digested genomic DNA of the generated cell lines are prepared or, alternatively, the blot is stripped after hybridization of the first probe and then hybridized to a second. Either method should allow overlay of the signals obtained with E1A and the E1B probes.
If, following transfection and integration during generation of the transformed cell line, the E1A fragment becomes integrated next to an E1B-containing fragment the probes for E1A and E1B detect the same bands on the blots, the size of the band identifying the distance between the two genes. Since the orientation of the E1A and E1B fragment relative to each other is not known, the two E1B probes are used separately enabling hybridization either to the fragment 5′ or 3′ of the BglII restriction site. If an E1A fragment becomes integrated next to another E1A fragment then the band resulting from EcoRV digestion will not be recognized by the E1B probe. Also single integrants or end fragments will generate bands that are not recognized by both probes.

Altogether, the experiments in this example clearly show that co-transfection of primary human cells with separate plasmids, one containing E1A flanked by a large stuffer region and one containing E1B flanked by backbone sequences, results in transformed cell lines. Furthermore, these cell lines express E1 proteins in quantities sufficient for efficient

complementation of E1-deleted Ad5 vectors.

In an alternative embodiment, the E1A and E1B sequences are cloned into a single construct, with a stuffer fragment between these sequences, after which cell lines are created using the single construct.

Clearly, if larger distances between E1A and E1B are desired, the E1B coding sequences may also be flanked by a longer stuffer nucleic acid, and/or the length of the stuffers flanking the E1A and E1B coding sequences could be increased, by standard routine molecular biology techniques, following the teachings of the present disclosure. Clearly therefore, this example should not be construed to limit the scope of the invention to the actually performed experiments, but rather as an exemplification of the concepts of the present invention.

Tables

Table II: Transformation of primary HER cells with Ad5-E1 expression constructs.

Construct	Amount	# dishes	foci/dish
pIG.E1A.E1B	20 µgr	2	31
pIG.E1A	20 µgr	2	0
pIG.E1A+pE1B	10 µgr each	4	15
pIG.E1AB21	20 µgr	2	11.5
pIG.E1AB21+pE1B	10 µgr each	4	10
AdApt.eGFP	20 µgr	1	0

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CLAIMS

1. A method of preparing a cell having adenovirus E1A and E1B-19K and E1B-55K coding sequences integrated into its genome, the method comprising the steps of introducing into a precursor cell:
a) a nucleic acid molecule comprising the E1A coding sequence and a separate nucleic acid molecule comprising the E1B-19K and E1B-55K coding sequences, wherein said nucleic acid molecules lack substantial homologous sequence overlap that could otherwise lead to homologous recombination between said molecules; or
b1) a single nucleic acid molecule comprising the E1A and E1B-19K and E1B-55K coding sequences, at least two of these three coding sequences being separated by at least 4 kb; or
b2) two or more nucleic acid molecules that when these would form a single nucleic acid molecule would form a single nucleic acid molecule according to b1).

2. A method according to claim 1, wherein a nucleic acid molecule comprising the E1A coding sequence and a nucleic acid molecule comprising the E1B coding sequences are introduced into the precursor cell, wherein said sequences lack substantial overlap that could otherwise lead to homologous recombination between said molecules.

3. A method according to claim 1 or 2, wherein said E1A and at least one of the E1B coding sequences are under control of a heterologous promoter and/or polyadenylation signal.

4. A method according to claim 1, wherein the introduced nucleic acid molecules comprise
a) a single nucleic acid molecule comprising the E1A and E1B-19K and E1B-55K coding sequences, at least two of these three coding sequences being separated by at least 4 kb; or
b) two or more nucleic acid molecules that when these would form a single nucleic acid molecule would form a single nucleic acid molecule according to a).

5. A method according to claim 4, wherein at least two of said coding sequences are separated by at least 10 kb.

6. A method according to claim 5, wherein at least two of said coding sequences are separated by at least 34.5 kb.

7. A method according to any one of claims 1-6, wherein the nucleic acid sequences encoding E1B-55K and E1B-19K are on different nucleic acid molecules or separated by at least 4 kb when introduced into said precursor cell.

8. A cell comprising adenovirus E1A and E1B-55K and E1B-19K coding sequences in its genome, characterized in that said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 4 kb in said genome.

9. A cell according to claim 8, wherein said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 10 kb in said genome.

10. A cell according to claim 10, wherein said cell lacks stretches of nucleic acid sequence wherein said E1A and both E1B coding sequences are separated by less than 34.5 kb in said genome.

11. A method for providing a cell comprising adenoviral E1 sequences, wherein said E1 sequences include E1A and E1B coding sequences, characterized in that said method includes a step of selecting cells having a genome that lacks inverted repeats comprising said E1 sequences.

12. A cell comprising adenovirus E1 sequences in its genome, wherein said E1 sequences include at least one functional copy of the E1A and E1B-19K and E1B-55K coding sequences, characterized in that said E1 sequences or part thereof are not present in the form of inverted repeats in said genome.

13. A cell according to claim 12, wherein said cell comprises at least two copies of said E1 sequences in its genome.

14. A cell according to claim 12 or claim 13, wherein said cell is further characterized in that said E1A and at least one of the E1B coding sequences are separated by at least 4 kb in said genome.

15. A cell according to claim 14, wherein said E1A and at least one of the E1B coding sequences are separated by at least 10 kb in said genome.

16. A cell according to claim 12, wherein the genome of said cell comprises one copy of said E1 sequences in its genome and has no sequences encoding pIX.

17. A method for generating a batch of recombinant adenovirus having a deletion in the E1 region, comprising the steps of:
a) introducing said recombinant adenovirus or its genome into a cell comprising E1 sequences of an adenovirus capable of complementing the deleted E1 sequences of said recombinant adenovirus;
b) culturing said cell and harvesting said recombinant adenovirus,
the method characterized in that said cell is a cell according to any one of claims 8-10 or 12-16.

18. The method according to claim 17, wherein said recombinant adenovirus lacks substantial sequence overlap with the E1 sequences present in said cell.

19. A recombinant molecule comprising nucleic acid sequences encoding adenoviral E1A protein and at least one adenoviral E1B protein, characterized in that said nucleic acid sequence encoding E1A protein and a nucleic acid sequence encoding at least one E1B protein are separated by at least 4 kb.

20. A recombinant molecule according to claim 19, wherein said nucleic acid sequence encoding E1A protein and a nucleic acid sequence encoding at least one E1B protein are separated by at least 10 kb.

21. A recombinant molecule according to claim 20, wherein said nucleic acid sequence encoding E1A protein and a nucleic acid sequence encoding at least one E1B protein are separated by at least 34.5 kb.

22. A recombinant molecule according to any one of claims 19-21, wherein said molecule is linear.

23. A set of at least two nucleic acid molecules comprising:
a) a nucleic acid molecule encoding E1A protein of an adenovirus, wherein said nucleic acid molecule has at least 5 kb of spacer nucleic acid sequence on both sides of said E1A coding sequence, said spacer nucleic acid not encoding both E1B proteins of an adenovirus; and
b) a nucleic acid molecule encoding an E1B protein of an adenovirus, wherein said nucleic acid molecule has at least 5 kb of spacer nucleic acid sequence on both sides of said E1B coding sequence, said spacer nucleic acid not encoding an E1A protein of an adenovirus.

24. A set of at least two nucleic acid molecules according to claim 23, wherein said spacer nucleic acid sequence is at least 17 kb.

25. A method of preparing a cell capable of complementing E1 deficient adenoviral vectors without generating helper dependent E1 containing particles, comprising the steps of:
a) introducing into a precursor or ancestor of the cell nucleic acid sequence(s) coding for E1A gene functions and E1B gene functions, or if the precursor cell already comprises one of E1A or E1B gene functions, the other of

the E1A and E1B gene functions; and

b) selecting or identifying cells having obtained E1A and E1B in a chromosomal configuration that prevents the formation of helper dependent E1 containing particles when the cells are used to complement a recombinant adenoviral vector deficient for one or more E1 gene functions, said vector lacking nucleic acid sequences that have substantial overlap with the chromosomally located E1 sequence that otherwise could give rise to homologous recombination.

26. A packaging system comprising a packaging cell having E1 sequences in its genome and a recombinant adenovirus vector with a deletion in the E1 region, wherein the genome of said vector lacks substantial overlap with the E1 sequences in the genome of said packaging cell, characterized in that said packaging cell is a cell according to any one of claims 8-10 or 12-16.